Proc. Natl. Acad. Sci. USA
Vol. 75, No. 1, pp. 285–288, January 1978
Biochemistry

Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide

(*in vitro* protein synthesis/nucleic acid hybridization/DNA nucleotidyltransferase)

MARY L. STEPHENSON AND PAUL C. ZAMECNIK

The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Massachusetts 02114

Contributed by Paul C. Zamecnik, November 10, 1977

ABSTRACT **A tridecamer oligodeoxynucleotide, d(A-A-T-G-G-T-A-A-A-A-T-G-G), which is complementary to reiterated 3′- and 5′-terminal nucleotides of Rous sarcoma virus 35S RNA, is an efficient inhibitor of the translation of proteins specified by the viral RNA in the wheat embryo cell-free system. The inhibition specificity for oncornavirus RNA is greater than for rabbit reticulocyte mRNA or brome mosaic virus RNA. Other oligodeoxynucleotides of similar size have little or no specific effect on the RNA-directed translation. The tridecamer acts as a primer for the avian myeloblastosis virus DNA polymerase when Rous sarcoma virus heated 70S RNA is used as a template, offering evidence that it can hybridize to the RNA. The possible use of such an oligodeoxynucleotide hybridization competitor to inhibit Rous sarcoma virus replication is described in the preceding paper [Zamecnik, P. C. & Stephenson, M. L. (1978) *Proc. Natl. Acad. Sci. USA.* 75, 280–284].**

A sequence of 21 nucleotides immediately adjacent to the 3′-terminal poly(A) in Rous sarcoma virus (RSV) 35S RNA has been determined in these laboratories (1), and the same nucleotide sequence has been found by others at the 5′-terminus of the RNA (2). Thus, the genome RNA for this oncornavirus, and as more recently determined for avian myeloblastosis virus (AMV), is terminally redundant (3–8).

We are exploring the possibility that an oligodeoxynucleotide [d(A-A-T-G-G-T-A-A-A-A-T-G-G)] that is complementary to 13 nucleotides of the reiterated terminal sequences of RSV, may act as a competitive inhibitor of hybridization and interfere with viral production and cell transformation. Likely sites of action are (*i*) the circularization step of the proviral DNA intermediate, and (*ii*) the initiation of translation. Such a tridecamer [d(A-A-T-G-G-T-A-A-A-A-T-G-G)] has been synthesized (Collaborative Research), and in the preceding paper (9) we show that the production of RSV is inhibited when the tridecamer is added to chick embryo fibroblast tissue cultures infected with RSV.

This paper is concerned with the effect of the complementary oligodeoxynucleotide on the translation of heated RSV 70S RNA in a cell-free wheat embryo system. We find that the synthetic tridecamer serves as an efficient inhibitor of the translation of denatured RSV 70S RNA and to a lesser extent denatured AMV 70S RNA, which has a somewhat similar reiterated sequence. The inhibition is greater for oncornavirus RNAs than for globin mRNA or brome mosaic virus (BMV) RNA. In addition, several oligodeoxynucleotides of the same size range have only a small effect on the translation stimulated by any of the mRNAs. The tridecamer efficiently primes the reaction catalyzed by AMV RNA-dependent DNA polymerase (reverse transcriptase, EC 2.7.7.7) using RSV heated 70S or 35S RNA as template, thus supporting the assumption that it will hybridize to regions on the viral RNA. It remains to be seen at what point in viral protein synthesis the tridecamer is specifically interfering.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Generous supplies of AMV, strain BAI from infected chick plasma, and purified reverse transcriptase were supplied by Joseph and Dorothy Beard through the courtesy of J. Gruber of the Office of Resources and Logistics, National Cancer Institute. Rous sarcoma virus, Prague strain C from infected tissue cultures, was obtained via the same auspices from Eugene Bernstein.

The tridecamer and blocked tridecamer (3′- and 5′-terminal hydroxyl groups as phenylisourea derivatives) were synthesized by John Hachmann, Collaborative Research, Inc. (9). Synthetic nucleotides were obtained from Collaborative Research. Labeled [^{3}H]dTTP (50 Ci/mmol) and [^{35}S]methionine (480 Ci/mmol) were from New England Nuclear. Unlabeled nucleotides were from Schwarz/Mann and the gel electrophoresis reagents were from Bio-Rad.

The wheat germ was from General Mills though the courtesy of W. C. Mailhot. Rabbit reticulocyte 9S RNA was a generous gift from J. Gilbert. We are grateful to P. Kaesberg for the BMV from which we extracted the total RNA.

RNA was isolated from AMV and RSV by phenol/sodium dodecyl sulfate extraction of frozen virus pellets, using minor modifications of our earlier procedure (10, 11). The RNA was fractionated on sucrose/Sarkosyl gradients and the 70S RNA was isolated. The 70S RNA was heated to 83° for 3 min and was used as such (Δ 70S RNA) or in some cases further fractionated on sucrose gradients to recover the 35S RNA (11). Total BMV RNA was extracted as above from a virus suspension and was not fractionated.

The wheat embryo protein-synthesizing system was essentially that of Roberts and Paterson (12) and Davies and Kaesberg (13), using a dialyzed 30,000 × *g* supernatant (see Table 2 for details).

The viral reverse transcriptase assay was standard (14, 15) using [^{3}H]dTTP and unlabeled dATP, dGTP, and dCTP with the viral RNAs as templates (see Table 1).

RESULTS

Ability of the tridecamer to prime the reaction catalyzed by purified AMV reverse transcriptase using high molecular weight viral RNA as template

The assumption that the synthetic tridecamer would hybridize with the reiterated regions of the viral RNA is supported by our

Abbreviations: AMV, avian myeloblastosis virus; RSV, Rous sarcoma virus; BMV, brome mosaic virus; Δ 70S RNA, 70S RNA heated to 83° for 3 min.

Table 1. Effect of tridecamer and blocked tridecamer on capacity to prime AMV and RSV heated 70S RNA, using purified reverse transcriptase

Addition	Amount, A_{260} units	Control cpm	AMV Δ 70S RNA (0.10 A_{260} unit) cpm	AMV Δ 70S RNA (0.10 A_{260} unit) cpm − control	RSV Δ 70S RNA (0.10 A_{260} unit) cpm	RSV Δ 70S RNA (0.10 A_{260} unit) cpm − control	RSV/AMV
Control		710 560	500		3,800		
Tridecamer	0.005		3,050	2,550	22,180	18,380	7.2
	0.02		3,340	2,840	26,600	22,800	8.0
	0.05	1,390	6,380	5,880	23,500	19,700	3.4
Blocked tridecamer	0.005		1,810	1,310	7,600	3,800	2.9
	0.020		3,410	2,910	10,600	6,800	2.3
	0.050	1,430	4,400	3,900	16,300	12,500	3.2
$(dT)_{12-18}$	0.020		103,600	103,100	32,900	29,100	0.28
	0.05	1,030	92,900	92,400	35,800	32,000	0.34
$(dT)_{12-18}\cdot(A)_n$	0.0006	150,800					

The viral reverse transcriptase assay and washing are similar to those used earlier (9, 14, 15) for different templates and primers. Each tube contained 100 μl, incubated for 90 min at 37°. The amounts of tridecamer and RNA templates were as indicated (1 A_{260} unit is the amount of material giving an absorbance of 1 when dissolved in 1 ml and the light path is 1 cm); in addition, the mixture contained 1 mM each dATP, dGTP, and dCTP, and [^{3}H]dTTP (0.2 mM containing 3×10^6 cpm), plus 1 μl of viral reverse transcriptase (7000 units/ml) (15). Aliquots (50 μl) were washed and measured for radioactivity (9).

finding that the tridecamer does act as a primer for purified AMV reverse transcriptase, using both RSV and AMV Δ 70S RNA or 35S RNA as template (Table 1). It is not surprising that the tridecamer is 7-fold better as a primer with RSV RNA than with AMV RNA as a template because it provides an uninterrupted sequence of 13 nucleotides for hybridization with RSV RNA but only 10 nucleotides for AMV RNA. The 3′-terminal sequence of AMV 35S RNA is similar to the reiterated sequence of RSV as found by Stoll *et al.* (8) and Dennis Schwartz in our laboratory (unpublished data). Stoll *et al.* have also demonstrated that AMV RNA has a reiterated sequence at either end. There are 3 nonhybridizing bases (two adjacent internal, one 3′-terminal) in the synthetic tridecamer DNA sequence complement to RSV RNA when compared with the reiterated termini of AMV.

Effect of tridecamer and of blocked tridecamer on cell-free translation

Inhibition of Translation of Oncornavirus High Molecular Weight RNA by the Tridecamer. The synthetic tridecamer inhibits translation of 70S Δ RNA of RSV and AMV in the wheat embryo system (Table 2 and Fig. 1). Several experiments show that the inhibition is concentration dependent. The tridecamer inhibition is greater for RSV message translation than for AMV translation. Marked inhibition occurs at a ratio of 0.005 A_{260} unit of tridecamer to 0.1 A_{260} unit of RSV RNA, and appreciable inhibition is present at 0.0005 A_{260} unit of tridecamer. At this lower level of tridecamer, the molar ratio of tridecamer to 5′- plus 3′-complementary RSV 35S RNA sequences is around 2:1, and at 0.005 A_{260} unit of tridecamer, there is a 20-fold excess of tridecamer. Radioautographs of sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis of labeled proteins indicate inhibition of synthesis of most if not all of the labeled proteins made in response to added viral RNA (Fig. 1).

Specificity of Inhibition by the Tridecamer. The tridecamer preferentially inhibits the translation by oncornaviral RNAs. The translation of RNA isolated from rabbit reticulocytes and from BMV RNA is inhibited by the tridecamer, but to a lesser extent than inhibition of the oncornavirus RNA-stimulated translation, and only at high ratios of tridecamer to RNA (Table 2). At low ratios (0.0005 A_{260} unit of tridecamer to 0.10 A_{260} unit of RNA) there is no inhibition of the globin or BMV RNA-stimulated incorporation. Table 2 indicates that there is slight inhibition of the endogenous translation by the tridecamer at high concentrations.

Comparison of Inhibition of Tridecamer with Other Similar-Sized Oligodeoxyribonucleotides. Comparison with two similar-sized oligodeoxyribonucleotides, $(dG)_{12-18}$ and $(dC)_{12-18}$, indicates that they do not inhibit the translation at a concentration level at which the tridecamer inhibits over 90% of viral RNA-stimulated incorporation. This is shown in the radiofluorogram of a gel in Fig. 1. In Fig. 1 comparison of bands 2, 5, 7, and 9 shows the specificity of tridecamer for inhibition of protein synthesis due to Δ 70S RNA, band 5 (tridecamer plus Δ 70S RNA) indicating almost complete inhibition compared with band 2 (Δ 70S RNA alone), while 7 and 9 indicate that the presence of oligo(dG) or oligo(dC) has little effect on the protein synthesized in response to Δ 70S AMV RNA (band 2). Two mixed polymers of 8 and 10 deoxynucleotides of other defined sequences (Table 2, compounds A and B) also failed to show the degree of inhibition observed by the synthetic complementary tridecamer.

Blocked tridecamer added to the wheat embryo system stimulated by Δ 70S RNA is less inhibitory than the unblocked tridecamer (not shown). This is not surprising, because the size of the terminal blocking groups (i.e., the phenylisoureas) may interfere with hybridization of the tridecamer on sites on the viral mRNA (9).

DISCUSSION

Evidence in the preceding paper (9) indicates that addition of the tridecamer to chick embryo fibroblast cells in culture inhibits production of RSV. The *in vitro* evidence presented here (Table 1) of the ability of the tridecamer to prime oncornavirus high molecular weight RNA, using purified reverse transcriptase, indicates that the oligodeoxyribonucleotide is able to hybridize with the viral RNAs, presumably on the 3′ member of the reiterated sequences. The tridecamer primes at least 7 times more efficiently with RSV than AMV RNA. In the experiment cited in Table 1, 0.1 A_{260} unit of the RSV 70S RNA was satu-

Table 2. Effect of oligodeoxynucleotides on the translation of various RNAs in the wheat embryo system

	Amount, A_{260} units	Endogenous control	% inhib*	AMV Δ 70S RNA cpm − control	% inhib*	RSV Δ 70S RNA cpm − control	% inhib	Globin mRNA cpm − control	% inhib*	BMV RNA cpm − control	% inhib*
		5,800		16,100		15,000		41,500		303,200	
		7,100		15,300		12,500		39,700		294,900	
		Average 6,500	—	15,700	—	13,800	—	40,600	—	299,100	—
Tridecamer†											
	0.05	4,500	31	1,100	93	200	99	21,500	47	109,500	63
	0.005	5,400	17	6,700	57	1,600	88	30,400	25	209,500	30
	0.0005	8,600	+32	14,200	10	10,100	27	39,300	3	297,500	0.5
Oligodeoxynucleotides†											
A	0.04	10,900	+67	15,100	4	10,800	22	43,100	+6	313,700	+5
	0.004	8,900	+40	14,800	6	12,300	11	41,500	+2	323,800	+8
B	0.03	8,900	+40	16,000	+2	5,800	58	27,400	33	291,600	3
	0.003	8,000	+23	20,300	+29	13,300	4	38,300	6	319,200	+7

The translation assay was a slight modification of standard procedures (12, 13) using a dialyzed S-30 (30,000 × *g* supernatant) wheat embryo extract. Conditions are optimized for AMV Δ 70S RNA. When used together with RNA, all the oligodeoxyribonucleotides were preincubated at 37° for 5 min in 20 μl of 0.05 M KCl, then chilled to facilitate annealing. Each assay tube contained 50 μl; 10 μl was used for measurement of radioactivity and the rest was used for gel electrophoresis. The incubation contained 0.10 A_{260} unit of the RNA, 2 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 2.5 μg of creatine kinase, 4.1 mM magnesium acetate, 0.08 mM spermine, 1 mM dithiothreitol, 55 mM potassium acetate, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer at pH 7.5, 0.032 mM of each amino acid except methionine, 0.70 μM [^{35}S]methionine (480 Ci/mmol), and 25 μl of dialyzed S-30. The cpm are of [^{35}S]methionine incorporated into alkali-labile, trichloroacetic acid-insoluble material following incubation at 30° for 60 min. After incubation, 250 μg of carrier albumin was added followed by 1.0 ml of 10% trichloroacetic acid containing 1 mg of methionine per ml. The precipitate was centrifuged and the pellet was dissolved in 1 ml of 0.2 M NaOH at 37° for 20 min to hydrolyze the RNA. Proteins were reprecipitated by the addition of 1 ml of 20% trichloroacetic acid with methionine as above. The tubes were chilled 20 min and the precipitates were filtered onto a glass-fiber filter (GF/C), 25-mm diameter, using a Hirsch funnel. Proteins were washed eight times with 10 ml of 5% trichloroacetic acid and once with 10 ml of 95% ethanol. The filters were dried, 75 μl of H_2O plus 0.5 ml of NCS solubilizer (Amersham/Searle) was added, and the mixtures were warmed to 60° for 1 hr. Ten milliliters of counting fluid was added [toluene/0.3% 2,5-diphenyloxazole (PPO)/0.03% 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP), Nuclear Chicago] and the radioactivities of the samples were measured in a Nuclear Chicago scintillation counter.

* +, % stimulation over the control value.

† Tridecamer is d(A-A-T-G-G-T-A-A-A-A-T-G-G); A is d(G-G-A-A-T-T-C-C); B is d(C-C-A-A-G-C-T-T-G-G).

rated by the primer at 0.005 A_{260} unit or below, while the AMV 70S RNA was not saturated by 10 times as high a concentration of primer. This is likely due to the uninterrupted complementarity of the tridecamer with RSV RNA, compared with AMV RNA, which contains three nonhybridizing bases.

Table 1 also indicates that tridecamer blocked at both ends (9) was 80% less efficient as a reverse transcriptase primer than unblocked primer at the lowest concentration used. Higher concentrations allowed more priming to take place. Such priming could be due to loss of blocking agent during the incubation or to the presence of small amounts of unblocked tridecamer in the preparation, which would have a free 3'-OH at the 3' end of the molecule where priming is initiated. Because no attempt was made to remove the 70S RNA-associated viral tRNA, which is the natural primer, other than to heat to 83° for 3 min to disrupt the annealing, there is a small amount of priming activity with the 70S RNA. In these experiments all four deoxynucleoside triphosphates were used, one of which ([^{3}H]dTTP) was labeled.

[α-^{32}P]dATP was used by our colleague, Dennis Schwartz, with RSV 35S RNA in similar experiments, also indicating good priming to roughly the same extent (unpublished data).

The evidence of hybridization of the synthetic tridecamer to the oncornaviral RNA supports the inference that such annealing may inhibit virus-specified DNA integration into the host DNA (9) or may interfere with translation of viral proteins.

We have previously found (unpublished experiments) that the wheat embryo system (12, 13) makes virus-specific proteins in response to Δ 70S RNA or 35S RNA as judged by the appearance of labeled proteins in antigen–antibody complexes. On sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis the [^{35}S]methionine-labeled proteins, though virus-specified, do not correspond with the few proteins found in the mature virion. This has been found by others using oncornavirus RNAs in the wheat germ system. These proteins are of various sizes (see Fig. 1, band 2 compared with 10). Whether this is due to early termination, limiting elongation, aberrations in processing, or the properties of the virion RNA itself is not yet known. The system is capable of making globin in response to reticulocyte globin mRNA (Fig. 1, band 3).

It remains to be determined whether this inhibition of translation by the tridecamer is due to hybridization of tridecamer near potential initiation site(s) for viral protein synthesis close to the 5' end of 35S RSV or AMV RNA, or to interference by structural effects on initiation sites farther from the 5' end. As for possible interference by preventing complexing of mRNA to ribosomes, because the nucleotide sequence at the 3' end of 18S RNA differs markedly from a complement to the tridecamer (20), the latter possibility does not appear likely. There was a slight inhibition of globin synthesis by the tridecamer when globin message was used. This may be explained by the fact that there is a partial sequence homology of the 3' terminal region of globin mRNA and the 3'-terminal nucleotides in RSV 35S RNA (1, 21).

Recent experiments (22) suggest that initiation may take place at several sites on the viral RNA. It has not been resolved, however, whether the sites are active within the intact viral mRNA or whether there are separate mRNAs for the group-specific antigen, polymerase, envelope, and sarcoma regions of the genome. There are several reports (see ref. 22) of intracellular virus-specific mRNA species of less than 35 S that are

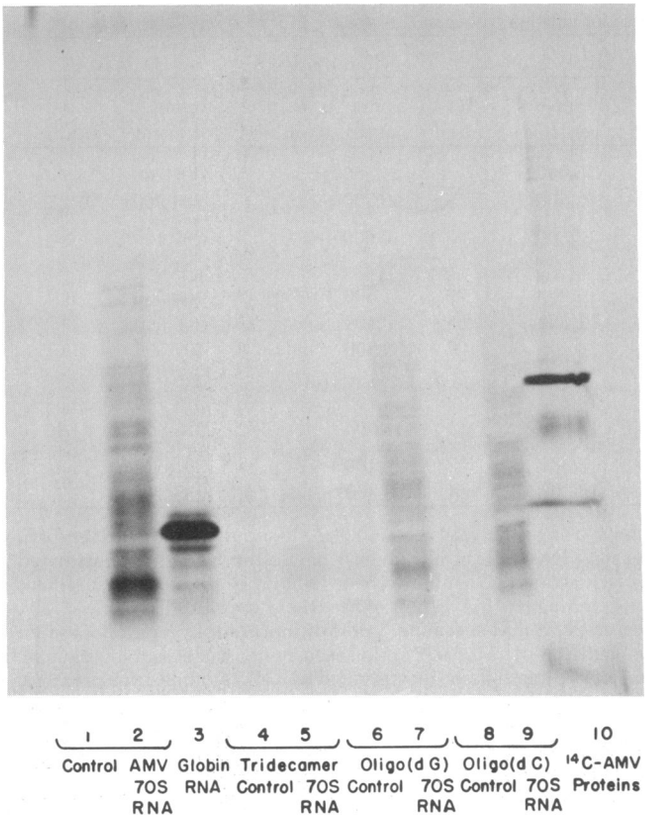

FIG. 1. Radiofluorogram of slab gel indicating inhibition by tridecamer of proteins synthesized in response to AMV Δ 70S RNA in the wheat embryo system. Sodium dodecyl sulfate (0.1%)/polyacrylamide (7–18%) slab gels (16 × 25 × 1.5 cm) of [^{35}S]methionine-labeled wheat embryo extract were run in the buffer system of Laemmli (16) with the gradient modified by Gielkens *et al.* (17). The enzyme assay conditions are given for Table 2. Aliquots (40 μl) of the indicated incubation mix were applied in each well. Marker ^{14}C-labeled purified AMV was prepared using [^{14}C]formaldehyde by the method of Rice and Means (18). Thirty micrograms of AMV containing 1400 cpm was added in well 10. Where indicated the incubation mix contained 0.08 A_{260} unit of AMV Δ 70S RNA, tridecamer, oligo(dC), and oligo(dG). Radiofluorograms were made of the gel (19), exposed at −70° for 3 days. A photograph of the film is shown. The major band of AMV in well 10 is the p27 structural protein.

apparently subsets of the 35S genomic RNA found in the mature avian tumor virus. The 70S RNA isolated from virions contains predominantly 35S RNA genomic material, but in addition we find there are smaller molecular weight RNAs that have mRNA activity, producing virus-specified proteins in the wheat embryo system.

The possible use of an oligodeoxynucleotide hybridization competitor to inhibit oncogenic viral production offers a mechanism of future chemotherapeutic potentiality, with possibilities for both RNA and DNA oncogenic viruses, and for other nonintegrating viruses as well.

It might also be possible to inhibit the translation of a specific cell protein, if that were desirable. The inhibition of globin synthesis might, for example, be useful in polycythemia vera.

The authors are grateful to Maryann T. Magner for excellent technical assistance. We also thank Dr. Dennis Schwartz and Dr. Jesse F. Scott for valuable discussions. This work was supported by Contract N01 CP 71007 of the Virus Cancer Program and by Contract EY-76-5-02-2404 of the Energy Research and Development Administration. This is Publication no. 1532 of the Harvard Cancer Commission.

1. Schwartz, D. E., Zamecnik, P. C. & Weith, H. L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 994–998.
2. Haseltine, W. A., Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 989–993.
3. Shine, J., Czernilofsky, A. P., Friedrich, R., Bishop, J. M. & Goodman, H. M. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1473–1477.
4. Coffin, J. M. & Haseltine, W. A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1908–1912.
5. Collett, M. S., Dierks, P., Cahill, J. F., Faras, A. J. & Parsons, J. T. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2389–2393.
6. Friedrich, R., Kung, H.-J., Baker, B., Varmus, H. E., Goodman, H. M. & Bishop, J. M. (1977) *Virology* **79,** 198–215.
7. Tal, J., Kung, H.-J., Varmus, H. E. & Bishop, J. M. (1977) *Virology* **79,** 183–197.
8. Stoll, E., Billeter, M. A., Palmenberg, A. & Weissmann, C. (1977) *Cell* **12,** 57–72.
9. Zamecnik, P. C. & Stephenson, M. L. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 280–284.
10. Stephenson, M. L., Wirthlin, L. R. S., Scott, J. F. & Zamecnik, P. C. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1176–1180.
11. Heine, U. I., Weber, G. H., Cottler-Fox, M., Layard, M. W., Stephenson, M. L. & Zamecnik, P. C. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3716–3720.
12. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2330–2334.
13. Davies, J. W. & Kaesberg, P. (1974) *J. Gen. Virol.* **25,** 11–20.
14. Baltimore, D. (1970) *Nature* **226,** 1209–1211.
15. Scolnick, E. M., Aaronson, S. A., Todaro, G. J. & Parks, W. P. (1971) *Nature* **229,** 318–321.
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
17. Gielkens, A. L. J., Van Zaane, D., Bloemers, H. P. J. & Bloemendal, H. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 356–360.
18. Rice, R. H. & Means, G. E. (1971) *J. Biol. Chem.* **246,** 831–832.
19. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
20. Shine, J. & Dalgarno, L. (1974) *Biochem. J.* **141,** 609–615.
21. Proudfoot, N. J. (1977) *Cell* **10,** 559–570.
22. Reynolds, F. H., Jr. & Stephenson, J. R. (1977) *Virology* **82,** 255–264.